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Immunohistochemical search for viral and bacterial antigens in Crohn's disease

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Abstract

Background: Recent studies show that diseased intestinal tissues of patients with Crohn's disease (CD) contain obstructed lymphatics, granulomas, and tertiary lymphoid organs, representing responses to persistent antigen.
Methods: Forty-seven tissue sections from 28 CD patients and 20 tissue sections from 17 control patients were studied. Tissues were immunostained with antibody directed against adenovirus, Epstein-Barr virus, herpes simplex virus I, parvovirus B19, *Listeria monocytogenes*, *Escherichia coli*, *Clostridium perfringens*, and *Mycobacterium avium* subspecies *paratuberculosis*.
Results: There was no evidence of adenovirus, Epstein-Barr virus, parvovirus B19, or *M. avium* subsp. *paratuberculosis* in the tissues. Clostridia were positively stained in the mucus of 18.5% of CD patients versus 35.3% of controls and in the tissue of 11.1% of CD patients but in no controls. Immunoreactivity to listeria antibody occurred in the mucus of 3.7% of CD patients and in 5.9% of controls while it occurred in the tissue of 37.0% of CD patients and 29.4% of controls. *E. coli* occurred in the mucus of 48.1% CD and 64.7% controls and in the tissue of 18.5% and 5.9% respectively.
Conclusions: Of the agents demonstrated in this search, none was located in granulomas or inflamed lymphatics. Finding the common gut microbes, *E. coli* and clostridia, in the mucus of patients and controls was not unexpected. The minor focal staining of *E. coli* and clostridia does not suggest a primary role for these pathogens in CD. Positive staining for listeria in patients and controls may very well represent cross reactivity rather than specific identification.

1. Introduction

Crohn's disease (CD) is a chronic enteric disorder characterized by segmental lesions along the intestine, most frequently the

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ileum and proximal right colon.[1] It is a transmural disease, with lymphocytic and granulomatous thickening of all four layers of the intestinal wall, mucosal ulcers, and fissures that extend from mucosa to serosa. In the submucosa and serosa, there is lymphocytic perilymphangitis, extensive obstructive lymphangitis, and chronic fibrosis.[1,2] Currently, the disease is thought to be caused by genetic susceptibility, dysbiosis of the flora of the gut, and inappropriate immune responses.[3] The "trigger" initiating the latter has not yet been identified. Recently there has been renewed attention to the role of lymphatic inflammation and obstruction. Pathologists have long believed the seat of anatomic alteration in CD to be the intestinal lymphatics.[4] Histologically, lymphoid aggregates and tertiary lymphoid organs (TLO) expand the submucosa and subserosa, and granulomas occur in all four layers.[2] These probably represent responses to persistent antigen. The characteristic TLOs and granulomatous disease suggest an infectious process, with perhaps a virus in the former, or a bacterium in the latter instance. The present study is a follow-up to the work of Sura et al. who demonstrated lymphocytic perilymphangitis with TLOs and granulomatous obstruction of lymphatics.[2] This study sought to demonstrate antigens of viral or bacterial origin in the diseased tissues.

Immunohistochemistry was employed using antibody against four viruses and four bacteria. Some of these were chosen for their known propensity to cause lymphangitis in other organ systems. These include adenovirus,[5] Epstein-Barr virus,[6] herpes simplex virus I,[7] and parvovirus B19.[8] A search for *Mycobacterium avium* subspecies *paratuberculosis* (MAP),[9–12] *Listeria monocytogenes*,[13] and *Escherichia coli*[14] was included because of continuing suggestions that they are important. *Clostridium perfringens* was studied because of its importance in the gut flora and over concern for its role as a secondary invader. This is a study similar to those of Cartun et al.[15] and Liu et al.,[16] however with emphasis on the lymphatics and granulomas.

2. Materials and methods

2.1. Patient tissues

Forty-seven tissue blocks from surgically resected portions of intestine (ileum and colon) from 28 CD patients were examined. These specimens originated from hospitals in France and the United States. Tissues were fixed in 10% formalin, processed through a graded series of alcohol and toluene, and embedded in paraffin. They were then cut at 4 μm and mounted on slides. Identical preparation was carried out on 20 tissue sections from 17 control patients. These were acquired from Hartford Hospital, Hartford, CT; Windham Hospital, Willimantic, CT; and the Department of Pathology, School of Medicine at Louisiana State University. They were obtained from surgical resections for neoplasias of the intestine and deaths by vehicular trauma. CD patients consisted of 12 males and 16 females ranging in age from 14 to 60 (mean age 28.6). Controls were 6 males and 11 females from 38 to 95 years of age (mean age 72.3). This study was approved by the Institutional Review Board of the University of Connecticut. Primary antibodies that were employed and procedural details for their use are listed in Table 1.

2.2. Positive control tissues

Case material known to be infected with the various viruses and bacteria was collected from the pathology archives at the University of Connecticut, and the Department of Pathology and Laboratory Medicine, Hartford Hospital. When infected tissues were not available, experimentally inoculated cell culture suspensions were concentrated in a clot of human plasma and thrombin, fixed in 10% formalin, and embedded in paraffin. These served as positive controls and for determining optimal antibody dilutions and epitope retrieval methods.

2.3. Immunohistochemistry

Tissue sections were deparaffinized, then pretreated by one of several methods to enhance staining by selected primary antibodies: Heat Induced Epitopes Retrieval for 20 min, pepsin digestion for 10 min, or proteinase K for 5 min (Table 1). For effective staining of the clostridia, tissues were treated by Heat Induced Epitopes Retrieval for 20 min, allowed to stand for 10 min, and treated for 4 min in protease XIV 0.08% solution in 0.05 M TBS pH 7 at 37 °C. (Procedure courtesy of Dr. J Smyth, Dept. Pathobiology & Veterinary Science, Univ. of Conn.)

Staining was carried out on a DAKO autostainer. Each time a series of slides was stained, a positive and negative (minus primary antibody) control was included. The automated steps were as follows. Three percent hydrogen peroxide was applied for 10 min to quench endogenous peroxidase activity, followed by a buffer rinse, DAKO protein block for 5 min, primary antibody for 60 min, and rinse with buffer. Secondary antibody (DAKO Envision+Dual Link polymer) was applied for 30 min followed by a buffer rinse. The slides were rinsed with DAKO Wash Buffer for 5 min prior to application of the chromagen (Vector NovaRed) for 10 min and a water rinse. Finally, the tissues were counterstained with DAKO hematoxylin for 5 min and rinsed with water. At the completion of the immunostaining, tissues were dehydrated through a graded series of alcohol and xylene, and coverslipped with Permount.

Observers were blinded to patient identity (CD vs. controls) during microscopic examination. Tissues were examined at 100 and 200×, studying contiguous vertical strips, mucosa (including attached mucus) through serosa (including mesenteric adipose tissue), left to right over the entire tissue section. Because of previous experience with D2-40 immunostaining of lymphatics and CD68 staining of granulomas,[2] these structures received special scrutiny. Granulomas numbered 1–12 per section; lymphatics were abundant, varied from section to section, and were often partially obstructed or dilated; they were not enumerated.

3. Results

There was no immunohistochemical evidence of adenovirus, Epstein-Barr virus, or parvovirus B19 in the tissues of either CD patients or controls. In the case of HSV I, one CD patient had positive immunoreactivity, occurring in a cluster of approximately 20 cells. Forty-six other slides from CD patients, including additional sections from the one positive